Effect of Glycerol Feed in Methanol Induction Phase for Hepatitis B Surface Antigen Expression in *Pichia pastoris* Strain KM71

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ABSTRACT

This study describes expression of HBs Ag in methylotrophic yeast, *Pichia Pastoris* under alcohol oxidase promoter. A single copy number of HBs Ag gene was transformed into *pichia* strain of KM 71, a Mut^s type, by using pA0815 pichia expression vector. The recombinant was cultivated in a shake flask either using methanol or a mixed feed of glycerol -methanol for induction. The HBs Ag gene integrity was justified using direct PCR method. The expressed products in the soluble cell extracts were analyzed by Western blot, SDS page, Bradford assay and ELISA tests. The recombinant HBs Ag was expressed successfully in *Pichia pastoris* strain KM71 at a high level of HBs Ag protein expression. Thus, an addition of glycerol in the ratio of glycerol per methanol 1/1 (g g^{-1}) consistently produced 2-fold increment in both biomass accumulation and HBs Ag productivity.

Key words: Biomass, Hepatitis B, KM71 strain, pAO815 expression vector, *Pichia pastoris,* Surface Ag

INTRODUCTION

Hepatitis B virus (HBV) is without any doubt one of the most common human viruses. Two billions of the six billions people alive today show evidence of past or current infection with HBV. Meanwhile, about 350 million people are chronically infected with hepatitis B virus (HBV) worldwide. Two-thirds of these patients develop liver cirrhosis resulting in portal hypertension, liver failure and hepatocellular carcinoma (Schumann *et al*., 2007). Chronic infection may lead to cirrhotic liver failure, while infected people have a 100-fold increased risk of developing hepatocellular carcinoma (Chisari, 2000). Nevertheless, infection with HBV has become a vaccine-preventable disease.

One of the most remarkable features of HBV is its production of surface-antigen particles (termed as HBs Ag) that are stabilized by disulfide bonds and contain carbohydrates and phospholipids (Dane *et al*., 1970; Robinson *et al*., 1974; Tiollais *et al*., 1985). By the early 1980s, advances in genetic engineering and biotechnologies allowed the first HB vaccine to be obtained through formulation of HBs Ag produced in the recombinant strains of the yeast *Sacharomyces cerevisiae* (Elliot *et al*., 1994). In line with *S. cerevisiae* based approach, the technology for the large-scale obtainment of HBs Ag was developed based on the expression of the HBs Ag gene under control of the *Pichia pastoris* alcohol oxidase I (*AOX1*) enzyme gene promoter (Eugenio *et al*., 2000).

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The methylotrophic yeast *P. pastoris* has been developed into a commercially important host for the production of heterologous proteins (Cereghino & Cregg, 2000; Lin Cereghina *et al*., 2001). The expression level was high in this eukaryotic *pichia* system, and most of the proteins are soluble (Shao *et al*., 2003). It is important to note that *pichia* is capable of growing at very high cell densities in a simple mineral medium. In fact, it does not have the endotoxin problem associated with bacteria or the viral contamination problem of proteins produced in animal cell culture.

Furthermore, *P. pastoris* can utilize methanol as a carbon source in the absence of glucose. High-level expression and efficient assembly of HBs Ag has been reported in *P. pastoris* by integrating a single copy of the HBs Ag gene under control of the AOX1 promoter (Cregg *et al*., 1987). In addition, multiple copy integration of recombinant HBs Ag genes in Pichia has also been demonstrated to increase the expression of a desired protein in the *Pichia* strain of GS115 (Vassileva *et al*., 2001). The aim of this study was to develop the genetically engineered *Pichia pastoris* system in Pichia KM71 strain system for the production of HBs Ag. The production would focus on the influence of glycerol feed during methanol induction phase.

MATERIALS AND METHODS

Strain and Vector

The *pichia* strain of KM 71, a histidine requiring auxotroph and the intracellular expression vector, pA0815 (Invitrogen, USA) were used in this study. The vector contained one unique restriction site of *Eco*R 1. A linearization of this vector with *Sal* I enzyme will generate His^+ Mut^s in KM 71.

Nucleotide Sequence of HBs Ag (681bp)

ATGGAGAACATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGG
TTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGG
ACTTCTCTCAATTTTCTAGGGGGATCTCCCGTGTGTCTTGGCCAAAATTCGC
AGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGG
TTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGC
TATGCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTT
GTCCTCTAATTCCAGGATCAACAACAACCAGTACGGGACCATGCAAAACC
TGCACGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTAC
AAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGC
TTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAG
TTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGG
CTTTCAGCTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCG
TGAGTCCCTTTATACCGCTGTTACCAATTTTCTTTTGTCTCTGGGTATACATTTAA

Cloning into Pichia Expression Vector

The HBs Ag of 681bp was initially amplified by PCR, from plasmid pEco63 (ATCC 31518; American Type Culture Collection, Rockville, Md.), which contains a full length of HBs Ag gene. The coding sequence of HBs Ag was amplified with the primers, designated as FHBs Ag (forward primer), 5'-G GAA TTC AAG CTT **ATG** GAG AAC ATC ACA TCA GG-3'(Invitrogen, California, USA) and RHBs Ag (reverse primer), 5'G GAA TTC AAG CTT **TTA** AAT GTA TAC CCA GAG AC-3' (Invitrogen, California, USA) (the *Eco*R1 recognition sites are underlined; the initiation codon in the forward primer and the reverse complement of the termination codon in the reverse primer are in boldface letters). PCR was carried out with 1 to 2 µl of the plasmid DNA in a mixture of 10x

PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 50*p*mol (each) sense and antisense primer, as well as 1 U of KOD HiFi DNA polymerase (Novagen, Germany) in a final volume of 50 µl. Amplification was performed for 30 cycles with denaturation at 94°C for 1min, annealing at 60°C for 30 sec, extension at 72°C for 1min and finally at 72°C for 7min. The PCR product was analyzed using 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Meanwhile, the amplified PCR product of 707 base pairs fragment of HBs Ag was gel purified using GeneClean (QBIOgene, California, USA). The pA0815 vector and the purified PCR product were double digested with *EcoR1* restriction enzyme at 37°C for 16 h. The vector was then treated with Antarctic phosphatase (New England Biolabs INC., Ipswich, UK) for ½ h at 37°C, and this was followed by inactivation at 65°C for 5min. Both the digested PCR product and the linearized vector were purified using phenol, followed by chloroform-isoamyl alcohol (24:1) and finally precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.4) and 2.5 volume of 95% ethanol. It was then incubated at -20°C for 15min and centrifuged at a high speed using micro centrifuge at 4°C for 15 min (22R, Hettich, Tuttlingen, Germany). The supernatant was discarded and the pellet was washed with 70% ethanol. The PCR product, together with the linearized vector, was then ligated at 14°C overnight. A total volume of 100 ng ligated DNA was chemically transformed into Top 10F' competent cell by heat shock at 42°C for 1 min, and followed by incubation at 37°C with agitation for 1 h. This was then spread onto ampicillin LB agar plates and incubated at 37°C overnight. About 20 colonies were selected on the next day and sub-cultured into ampicillin LB broth. The recombinant plasmids were extracted by using a conventional plasmid extraction method.

The recombinant plasmids were verified by PCR for the correct direction of the gene inserted, using FHBs Ag as forward primer, with RAOX1 (5'- GCA AAT GGC ATT CTG ACA TCC-3') as reverse primer, and/or FAOX1 (5'-GAC TGG TTC CAA TTG ACA AGC-3') as a forward primer with RHBs Ag reverse primer. The selected positive clones were sub-cultured (see Figure 1) and purified using Concert High Purity plasmid purification system (Marligen 'Bioscience, USA).

In Vitro Multimerization

The size of the vector, containing one copy gene insert, was 8417bp in length. It was then digested with enzyme *Bgl*11 and *BamH* 1 for 16h at 37 °C. The digested product was analyzed using 1% agarose electrophoresis. The band size of 1980bp (containing one copy gene cassette) was excised from the gel, and this was followed by gel purification using GeneClean (QBIOgene, California, USA). This particular vector was also digested with *Bgl* 11 and *Bam*H 1 at 37 °C for 16h and dephosphorylated for ½h at 37 °C. Then, the DNA was extracted using phenol as described above. After that, the one copy gene cassette and the linearized vector were ligated at 14 °C overnight. The ligated DNA was chemically transformed into Top 10F' competent cell as mentioned in the discussion above. On the following day, about 20 colonies were selected and sub-cultured into the ampicillin LB broth. The recombinant plasmids were extracted using the conventional plasmid extraction method and also verified by PCR using the primer as mentioned above and double enzyme digestion by *Bgl* 11 and *BamH* 1 to determine the correct direction of the gene insert. The selected positive cloned was sub-cultured and purified using Concert High Purity plasmid purification system for the subsequence transformation step in *Pichia*.

Transformation into Pichia

The selected plasmid without gene insert pA0 (as negative control), and plasmid with one copy gene insert (pA1) were sub-cultured and purified. The total amount of 10µg DNA was digested with *Sal* 1 enzyme at 37 °C for 16h, followed by dephosphorylation. The linearized DNA was phenol

extracted as mentioned above. A total of 5µg of linearized DNA was prepared to be transformed into KM 71 *pichia* competent cell. The DNA was mixed with 40µl of *pichia* competent cell for 3 min. It was then transferred into a 2 mm electroporation cuvette, incubated for 5 min on ice and pulsed once at 1800 volt (EC 100 Electroporator, Krackeler Scientific, Inc., Albany, New York, USA). After the electroporation, 1 ml of ice-cold sorbitol was immediately added, and incubated for 1h and 30 min without shaking. Later, it was spread onto RDB (1M Sorbitol, 2% dextrose, 1.34% YNB, 4 x 10-5% biotin, 0.005% amino acids & 20g/L agar) plates and incubated at 30°C. The recombinant *pichia* colonies were analyzed for HBs Ag gene through direct colony PCR using the primers of FAOX1 and RAOX1.

PCR Screening of Pichia Transformants

The KM 71 *pichia* colonies were analyzed for the presence of the HBs Ag gene insert through direct colony PCR using the primers of FAOX1 and RAOX1. Briefly, a pinpoint sized part of a single colony was picked from a master plate and suspended in 10µl distilled water. Later, 5µl zymolase (1mg/ml of 60 000 unit zymolase) was added into it, mixed and incubated at 30°C for 10 min. The mixture was immediately frozen at –80°C for 10 min. After the lysis step, 1 µl of the mixture was template used for the PCR using the hot start method. The PCR reactions were 5 µl of 10x PCR buffer, 2.5 µl of 50mM $MgCl_2$, 2.5 µl of 10mM dNTP and 50*p*mol primer each. The reaction mixture was allowed to go for 95°C for 5 min and this was followed by adding the 2 U of *Taq* polymerase (New England Biolabs ING., Ipswich, UK). The positive clones were then sub-cultured onto MD (1.34% YNB, $4x10^{-5}$% biotin, 2% dextrose) agar plates.

HBs Ag Production in Shack Flask

A single colony from the MD agar was inoculated into 100ml of BMGY media (1% yeast extract, 2% peptone, 100mM potassium phosphate, pH 6.0, 1.34% YNB, $4x\ 10^{-5}$ % biotin, 1% glycerol) in a 500 ml baffled flask, and cultured at 30°C in a shaking incubator (300 rpm) until culture reached OD_{600}=2 (20h). The cells were then harvested by centrifugation at 1500x g for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in a 100 ml BMMY medium (the same as BMGY, except that the glycerol was replaced with 0.5% methanol) in a 500 ml baffled flask. During the induction phase, 100% methanol and 1g glycerol/g were added into the culture on a daily basis and to a final concentration of 1% so as to maintain the induction for the methanol fed culture. At each time before induction, samples of the cell culture were collected to determine the OD and dry cell weight until Day 13. In addition, the cell samples were also collected daily up to Day 13, while the culture was harvested by centrifugation at 5000g for 20 min at 4 ºC. The cell pellet was recovered and stored at –80°C until it ready for assay.

Optical Density

The OD was determined at 600 nm using a biophotometer (Eppendorf, Hamburg, Germany). The samples were diluted by PBS in an appropriate dilution.

Dry Cell Weight

A total volume of 2 ml (in duplicate) of the *pichia* culture was pelleted in a pre-weighed eppendorf microcentrifuge tubes by low centrifugation 5000x *g*, 10min, washed twice with PBS and centrifuged at 5000x *g* for 5 minutes. The pellet was dried at 80°C with a drier (Memmert, Braunschweig, Germany) and weighed after 1 day.

Preparation of the Cell Extract

A total volume of 1 ml of the *pichia* culture was pelleted by low centrifugation (5000x *g*, 10 min) and washed twice with PBS to remove the medium. The washed cells were resuspended in a 200µl lysis buffer (10mM sodium phosphate buffer, pH 7.2; 5mM EDTA, 0.5M NaCl, 0.1% (v/v) Triton X-100, 1mM PMSF), together with an equal volume of acid wash glass bead. The cell was lysed by 10 times vortexing using VX100 (Vortex Mixer, Labnet, NJ, USA) per min at the maximum speed and each was followed by chilling on ice for 1 min. Next, it was centrifuged at the maximum speed (12000 x g for 20 min) to collect the supernatant for Bradford, SDS page, Western blot and ELISA assays.

Bradford Assay

The dye reagent was diluted in 1-part dye reagent and 4-part double distilled water. Three to five dilutions of protein standard were prepared (from 2 mg/ml to approximately 10 mg/ml). Meanwhile, 10 µl aliquot standard and sample solution were added to separate microtiter wells. Then, a volume of 200 µl diluted dye reagent was added to each well. The microtiter plate was incubated for at least 5 min and the results were determined by reading at a wavelength of 590 nm using the ELISA reader (Tecan, Sunrise, Melbourne, Australia).

SDS PAGE Analysis

Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) was prepared according to Sambrook and Maniatis **(**1989) to determine the molecular weight of the expressed protein. The resolving gel, containing 12% acrylamide, 0.1% SDS and 0.375M Tris HCL (pH 8.8), was prepared. In addition, ammonium persulphate was also added, and this was followed by adding TEMED before it was allowed to solidify. The stacking gel, containing 4.75% acrylamide, 0.1% SDS and 0.125M Tris-HCL (pH6.8), was also prepared. The prepared cell lysate was mixed with a loading buffer (8% SDS; 50% β-ME; 1 M DTT, pH 6.8) and denatured by boiling for 10 min. The lysate was later loaded into the gel. After the electrophoresis of about 2 hrs, the gel was stained with Coomassie blue. After distaining, the bands were visualized using the gel documentation system (Alpha Innotech Corp., California, USA).

Western Blot Analysis

To analyse the expression of HBsAg, cell lysates were appropriately diluted with 1x SDS sample disruption buffer. After denaturation, the samples were loaded onto SDS gel and proteins were separated by gel electrophoresis. After the electrophoresis, the proteins were transferred to PVDF membrane (Immobilon-P^{SQ}, Millipore corp., Billerica, MA, USA). The non-specific binding sites were blocked using a blocking buffer (BSA 1%). The antigen-bound antibodies on the membrane were detected using HBs Ag primary monoclonal antibody and goat anti-mouse secondary antibody IgG HRP (Bio-Rad, California, USA). Finally, the signals were developed using the Western blotting ABTS substrate and the bands were visualized.

Dot Blot Analysis

The PVDF membrane (Immobilon-PSQ, Millipore Corp., Billerica, MA, USA) was wetted with methanol for 15 sec and rinsed with ultrapure water. After that, the membrane was placed in PBS for 5 min. About 5 µl of the sample was spotted onto the membrane and air dried. The dried membrane was rewetted with methanol for 5 min. Next, the membrane was placed into a blocking solution

(PBS; 1% BSA; 0.05% Tween 20). The mouse monoclonal antibody against HBs Ag (Biodesign, Phoenix, Arizona, USA) was diluted in 1000 folds and was added onto the membrane for 2 h. Then, the membrane was washed with PBS. The goat anti-mouse secondary antibody IgG HRP (Bio-Rad, California, USA) was diluted about 5000 folds, added onto the membrane and incubated for 1 hr. Later, the membrane was washed with PBS. Then, the DAB substrate was added onto the membrane and incubated for 10 min. The reaction was finally stopped with distilled water.

Enzyme Linked Immunosorbent Assay (ELISA)

The concentration of the protein HBs Ag in the soluble cell extracts was determined using the SURASE B-96 ELISA kit (General Biologicals Co., Taiwan). The kit was modified and re-optimized so that it could be used for a quantitative of HbsAg from the *pichia* cell extract. In particular, the *pichia* of pA0 was used as a negative control (vector without gene insert). Meanwhile, pure HBs Ag was used as a standard and to construct a standard curve using a serial dilution of commercial HBs Ag (Chemicon, Temecula, CA, USA) (0- 10 ng). The cell extracts of pA1 were diluted in 500 folds with a lysis buffer. As described by the manufacturer, 50 µl of the samples were then added into the wells and 50 µl guinea pig antibody (anti HBs) IgG HRP conjugated was also added and incubated for 1 hr. After that, it was washed three times. The TMB solution was added into the wells and incubated for 30 min. The reaction was stopped with 2 N H2SO4 (Fisher Scientific, Loughborough, UK) and read at a wavelength of 450/650 nm using the ELISA reader (Tecan, Sunrise, Melbourne, Australia).

RESULTS

The single copy number of HBs Ag gene was successfully cloned into the *pichia* expression vector pA0815 and designated as pA1. The PCR and DNA sequencing analysis of the pA1 indicated a correct orientation of HBs Ag gene insertion (see *Fig.1*). Meanwhile, the DNA sequencing results revealed 100% matching to the 681bp of HBs Ag encoding 226 amino acids. The recombinant vector pA1 digestion, with *Bgl*ll and *Bam*H1 restriction enzymes, produced three fragments of 4.2 kb, 2.3 kb and 2 kb. The HBs Ag gene resided in the 2 kb fragment.

The Coomasie blue stained SDS page gel demonstrated 23kDa band of HBs Ag from the *pichia* cell lysate (*Fig. 2*). In addition, Pichia HBs Ag particles have dimers of disulfide-bonded polypeptides with a molecular mass of about 23kDa. The dimer and oligomer showed 46 kDa and 89 kDa bands (see *Fig. 2*). The monomer (23KD), dimer (49KD) and oligomer (89KD) bands in the Western blotting (Figure 3), as well as the positive result obtained in the dot blot (see *Fig. 4*), have been confirmed to produce HBs Ag protein from the cell lysate. Furthermore, ELISA was also used to quantify the HBs Ag expression from the *pichia* recombinants. The technique was optimized using a standard curve from 0-10 ng/ml prepared using commercial HBs Ag so as to enable the determination of HBs Ag production level in pA1 pichia recombinant HBs Ag (see *Fig. 5*).

Prior to cultivation in the shake flask, the KM71 Mut^s was initially cultured in the MD agar to isolate the recombinants. The cultures grown in the BMGY media were replaced at day-3 with fresh BMMY containing either glycerol /methanol at 0/1 ratio (g g^{-1}) or a mixed feed of glycerol/ methanol at 1/1 ratio (g g^{-1}) for expression induction. The samples were collected until day-13 of cultivation. The results obtained from ELISA revealed that the production of HBs Ag expression started at day-1 of the induction until day-10. Meanwhile, the bio-mass DCW achieved at day-10 (day 6 of induction) was 9.4 g/l in glycerol /methanol at 0/1 ratio (g g^{-1}) and 21 g/l for glycerol / methanol at 1/1 ratio (g g^{-1}) fed culture, respectively. The maximum production level of HBs Ag was achieved on day-10 but this gradually and subsequently declined for both the induction cultures (*Fig. 5*).

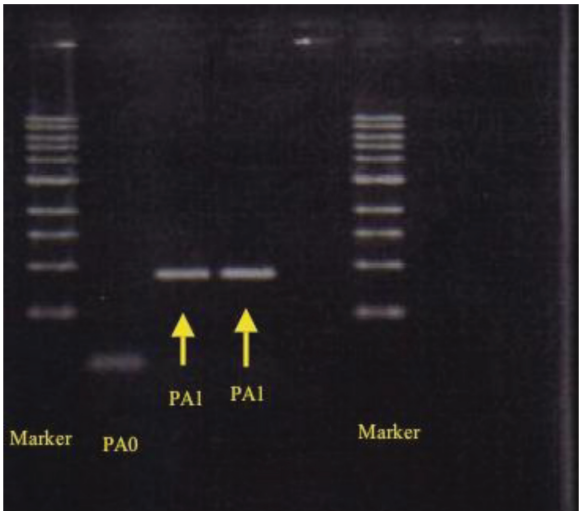
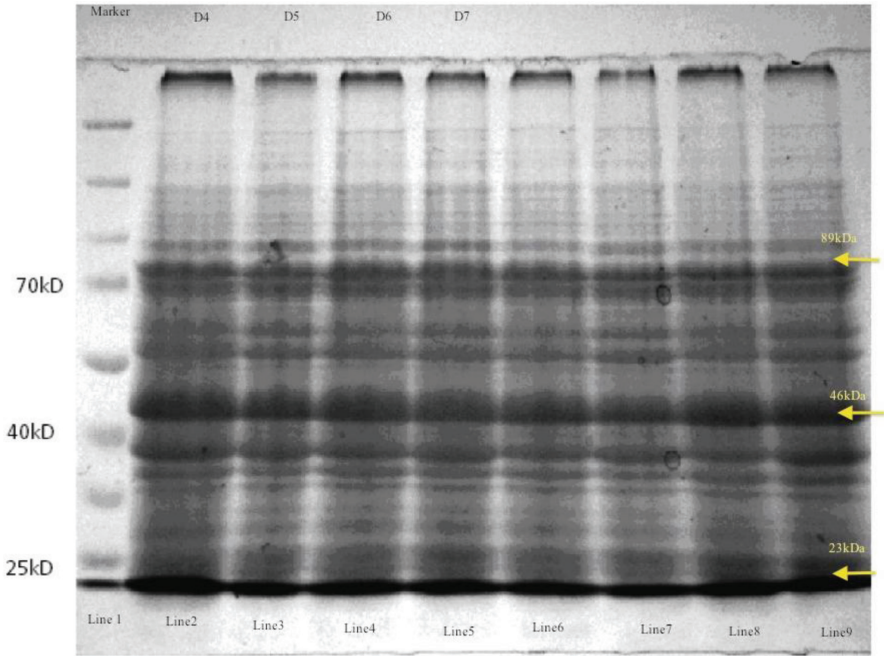


Fig. 1: A PCR analysis of the Pichia KM 71 recombinant. Lane 1: 1 kb DNA Marker. Lane 2: pA0 negative control recombinant KM 71(7700 kb). Lanes 3 and 4: Arrows indicate pA1, recombinant KM 71 that contained one copy of gene insert (8417 kb). Line 5: Empty. Lane 6: 1 kb DNA Marker



*D: Days

Fig. 2: The production of recombinant HBs Ag protein in KM71 strains (PA1) was analyzed by SDS page. The data showed that HBs Ag produced on Days 4 to 7 of the cultivation. Lane 1: protein marker. Lanes 2 to 5: KM71 pA1 culture in BMMY from Days 4 to 7. Lines 5 to 9 are repeated. They are fed with 0.5% methanol daily. The monomers protein size is about 23kD. The bands (Arrows) corresponding to dimer (46 kDa) and oligomer (89 kDa) are present as well as monomer (23 kDa)

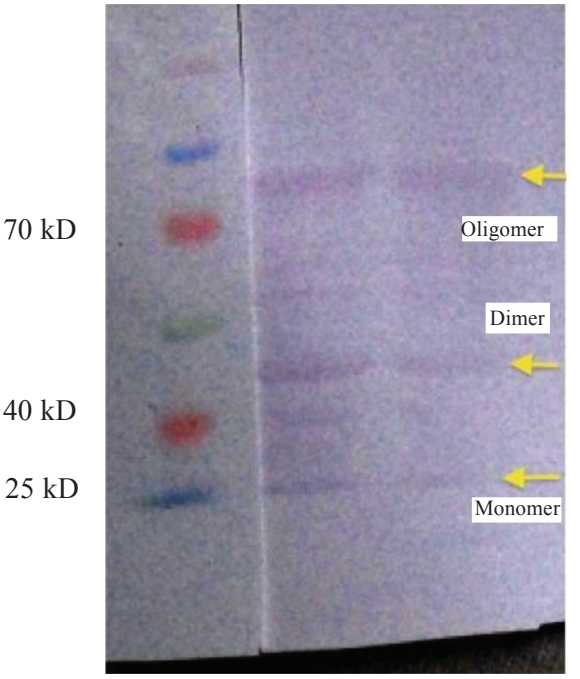
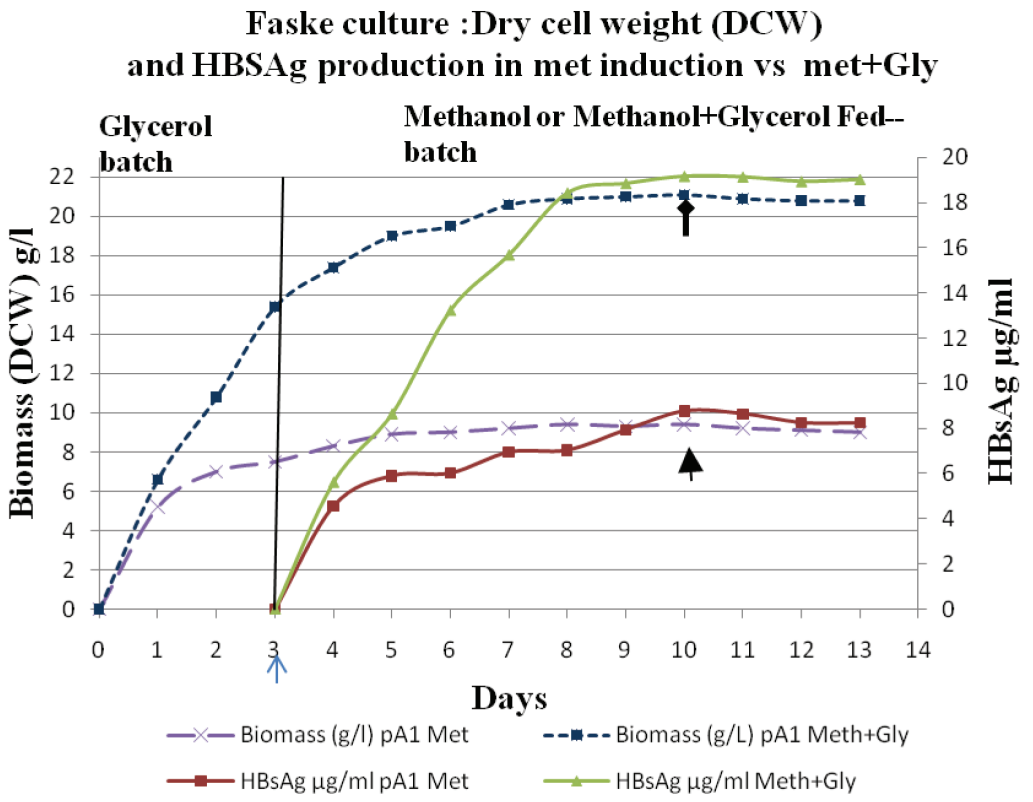


Fig. 3: The Western blotting analysis of the recombinant HBs Ag protein in the KM71 strain with anti HBs. The monomers, dimers and oligomers bands were confirmed by the Western blot with anti-HBs mAb

Fig.4: The dot blot analysis of the recombinant KM 71 showed a positive signal. The left arrow showed the positive result confirming that the HBs Ag protein was expressed in Pichia



*Met: Methanol Gly: Glycerol

Fig. 5: The recombinant KM71 strain pA1 in shake flask culture. The induction phase was started at ↑ Day 3 (Blue arrow). The biomass achieved 9.4 g/l (indicated by the thick arrow) in the glycerol /methanol at 0/1 ratio (g g^{-1}) and 21 g/l (indicated by thin arrow) for glycerol/ methanol at 1/1 ratio (g g^{-1}) fed cultures at Day 10. The expression of HBs Ag started at Day 4 and it achieved the highest level at Day 10 (day 7 of induction) in pA1 (see arrows)

DISCUSSION

All *Pichia* expression strains are derivatives of NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL); these examples included GS115, KM 71, MC100-3, SMD 1168, SMD 1165 and SMD 1163. Most of them have mutation in the histidinol dehydrogenase gene (*HIS4*) upon transformation (Cregg *et al*., 1985). Therefore, they need complex media with histidine supplementation. It is crucial to note that there are three types of host strains available. These vary according to their ability to utilize methanol which resulted from deletion in one or both *AOX* genes. The strains with deleted *AOX* genes are sometimes better producers of a foreign protein in corporation as compared to wild-type strains (Cregg *et al*., 1987; Chirulova *et al*., 1997; Tschopp *et al*., 1987). Moreover, these strains also require much less methanol to induce expression, which can be useful in large fermentor cultures. However, the most commonly used expression host is GS115, which is a wild type with regard to the *AOX1* and *AOX2* genes, and it grows on methanol at the wild-type rate (Mut^+). Meanwhile, in KM 71 *pichia* strain, the chromosomal *AOX1* gene is largely deleted and replaced with *S. cerevisiae ARG4* gene (Cregg & Madden, 1987). Therefore, it

must rely on the much weaker *AOX2* gene for *AOX* and grow on the methanol at a slow rate (Mut^s). It has been suggested that a fast growing Mut^+ strain was not as efficient as Mut^s in producing HBs Ag particles (Ottone *et al*., 2007). The activity of the AOX1 promoter in the methanol grown cells is significantly higher than the glucose grown cells with GAP promoter (Sears *et al*., 1998).

Pichia pastoris uses glycerol as carbon source during the initial cultivation and then utilizes methanol as a carbon source in the methanol fed-batch phase by converting it into formaldehyde. However, formaldehyde accumulation can inhibit cell growth. In the initial step of fermentation in the shake flask, the cells are initially grown on the media with glycerol (BMGY). In the second phase, the medium with glycerol (BMGY) would be replaced with the medium containing 0.5% of methanol (BMMY). In the final phase (induction phase), either the ratio of glycerol /methanol at 0/1 ratio (g g^{-1}) or glycerol /methanol at 1/1 ratio (g g^{-1}) was added into the culture. In a standard fermentation of Mut^S strain, the methanol fed rate should not exceed 0.3% (Invitrogen, 2000). Hence, the AOX promoter was induced to produce protein in both the fed-batch cultures.

Some previous studies have shown that a mixture of glycerol/methanol can be continuously added into *pichia* culture in various ratios (Ottone *et al*., 2007). The finding indicated a significant improvement in the cell culture viability and cell density upon induction with glycerol/methanol at 1/1 ratio (Table 1). A 2.3 fold biomass increment was produced as compared to the methanol induced cells in the shake flask method. In particular, the biomass in the shake flask reached 9.4 g/l and this was 21 g/l in the dry cell weight in methanol and in the mixed glycerol per methanol 1/1 (g g^{-1}) inductions, respectively (Table 1). Using a similar approach, Ottone *et al.* (2007) achieved 1.2 fold higher biomass accumulations in a bioreactor system. There are two types of biomass determination, namely dry cell weight (DCW) and wet cell weight (OD_{600} measurement). However, the dry cell weight (2.3 fold) measurement is more accurate than OD_{600} (1.8 fold) because during yeast budding, not all new cells get separated from the parent cell after division and they may stick together even until the next division. Thus, OD_{600} measurement does not measure the exact amount of cell density (Invitrogen, 2000).

In this study, a direct correlation of biomass increment with HBs Ag production (2.2 fold increment) was achieved as the transformant had a single copy number of integrant (pA1). Nonetheless, many fold increment of HBs Ag can be obtained with a multicopy integrant and

TABLE 1
A comparison of the expression of HBs Ag in the Mut^S strain (KM71) in a shake flask. Glycerol/ methanol mixed feed during the induction phase. A 2.3-fold biomass increment was produced when glycerol was added with methanol in the induction phase. Consequently, total protein and HBs Ag product were increased by increasing the biomass

Parameters	PA1 (single copy)	
	Glycerol/methanol 0/1 (g g^{-1})	Glycerol/methanol 1/1 (g g^{-1})
Dry cell weight (DCW g/l)	9.4	21
Wet cell weight (WCW g/l)	40	73
Final OD_{600}	46.5	86
HBs Ag (mg/l)	8.719.1	19.1
Specific yield mg HBs g^{1}DCW	0.92	0.91
Specific yield mg HBs g^{-1}WCW	0.21	0.25
Total protein	6.2	13.51

*DCW: Dry Cell Weight WCD: Wet Cell Weight

bioreactor application (Ottone *et al*., 2007). The finding derived at in the current study could be considered as significantly high in terms of the total HBs Ag expression level using a single copy integrant as compared to that of the previous study (Vassileva *et al*., 2001). In more specific, the results obtained in the present study demonstrated a 40.6 folds higher HBs Ag production (3.48 mg/100 OD cells) in the shake flask system using a single copy HBs Ag gene.

CONCLUSION

The HBs Ag has been successfully expressed in the *Pichia* strain KM71 recombinant. Consequently, a higher expression level of HBs Ag could be enhanced in the *Pichia* KM 71 strain by mixing feed methanol/glycerol at the 1/1 ratio (g g^{-1}) for induction.

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